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PATENT- OG VAREMÆRKESTYRELSEN

Light induced immobilisation

Field of the invention

The present invention relates to a method of cross-linking or immobilising proteins on a carrier.

Background of the invention

Molecules can be immobilised on a carrier or solid surface either passively through hydrophobic or ionic interactions, or covalently by attachment to activated surface groups. In response to the enormous importance of immobilisation for solid phase chemistry and biological screening, the analytical uses of the technology have been widely explored. The technology has found broad application in many different areas of biotechnology, e.g. diagnostics, biosensors, affinity chromatography and immobilisation of molecules in ELISA assays. The value of immobilisation technology is demonstrated by the recent development of DNA microarrays, where multiple oligonucleotide or cDNA samples are immobilised on a solid surface in a spacially addressable manner. These arrays have revolutionised genetic studies by facilitating the global analysis of gene expression in living organisms. Similar approaches have been developed for protein analysis, where as little as one picogram of protein need be bound to each point on a microarray for subsequent analysis. The proteins bound to the microarrays, can then be assayed for functional or structural properties, facilitating screening on a scale, and with a speed, previously unknown. The biomolecules bound to the solid surface may additionally be used to capture other unbound molecules present in mixture.

Development of this technology, with the goal of immobilising a biomolecule on a solid surface in a controlled manner, with minimal surface migration of the bound moiety and with full retention of its native structure and function, has been the subject of intensive investigation in recent years (Veilleux J (1996) *IVD Technology*, March p. 26-31). The simplest type of protein

immobilisation exploits the high inherent binding energy of surfaces to proteins in general. For example proteins will physically adsorb to hydrophobic plastic substrates via numerous weak contacts, comprising van de Waals, hydrophobic and hydrogen bonding interactions. The advantage of this method is that it avoids modification of the protein to be bound. On the other hand, proteins bound in this manner may be inactivated and/or distributed unevenly over the solid support, and their clustering may lead to steric hindrance in any subsequent functional assays.

Alternative methods of immobilisation rely on the use of a few strong covalent bonds to bind the protein to the solid surface (Wilson D.S., Nock S., 2001, *Current Opinion in Chemical Biology* 6:81-85). Examples include immobilisation of biotinylated proteins onto streptavidin-coated supports, and immobilisation of His-tagged proteins, containing a poly-histidine sequence, to Ni^{2+}-chelating supports. Other functional groups on the surface of proteins which can be used for attachment to an appropriate surface include reacting an amine with an aldehyde via a Schiff-base, cross-linking amine groups to an amine surface with gluteraldehyde to form peptide bonds, cross-linking carboxylic acid groups present on the protein and support surface with carbodiimide, cross-linking based on disulfide bridge formation between two thiol groups and the formation of a thiol-Au bond between a thiol group and a gold surface.

Amine coupling is a widely used method of immobilisation chemistry. N-hydroxysuccinimide esters are formed from a fraction of the carboxyl groups of the carboxymethyldextran matrix via reaction with N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) in water, which then react spontaneously with amine groups on a protein to form covalent bonds (Johnsson B., *et. al.*, 1991, *Anal Biochem* 198:268-77). Following immobilisation, un-reacted N-hydroxysuccinimide esters on the support are deactivated with 1M ethanolamine hydrochloride to block areas devoid of bound proteins. The method is laborious since the

reagents, used at each step of a chemical immobilization method, usually need to be removed prior to initiating the next step.

Methods for the immobilization of biomolecules via disulfide bridges are described by Veilleux J (1996) *supra*. Protein samples are treated with a mild reducing agent, such as dithiothreitol, 2-mercaptoethanol or tris(2-carboxyethyl)phosphine hydrochloride to reduce disulfide bonds between cysteine residues, which are then bound to a support surface coated with maleimide. Alternatively primary amine groups on the protein can be modified with 2-iminothiolane hydrochloride (Traut's reagent) to introduce novel sulfhydryl groups, which are thereafter immobilized to the maleimide surface. Immobilization of proteins on a gold substrate via a disulfide bridge is shown for the cupredoxin protein plastocyanin from Poplar (Andolfi, L. *et al.* 2002, *Arch. Biochem. Biophys.* 399: 81-88). Since this protein lacks a disulfide bridge, surface exposed residues Ile21 and Glu25 were both substituted with Cys. Disulfide bridge formation between the inserted cysteines was confirmed from the 3D crystal structure of the purified mutant plastocyanin. Mutant plastocyanin, expressed intracellularly in bacteria, is exposed to a reducing environment in the cytoplasm, such that the inserted cysteines are reduced, and can thus mediate the direct adsorption of the isolated protein onto a gold substrate. The thiol group binding properties of the protein are thus dependent on *in vivo* or *in vitro* chemical reduction of the cysteine residues on the surface of the protein.

An alternative approach to engineering thiol-group binding properties into a protein has been described for ribonuclease (RnaseA), which has four essential cystines (Sweeney, R.Y. et al. 2000 Anal Biochem. 286: 312-314). In this case a single cysteine residue was substituted for Ala19, located in a surface loop near the N-terminus of RNase A. The cysteine in the expressed RNase was protected as a mixed disulfide with 2-nitro-5-thiobenzoic acid. Following subsequent de-protection with an excess of dithiothreitol, the RNase was coupled to the iodoacetyl groups attached to a cross-linked agarose resin, without loss of enzymatic activity. Again, preparation of the

protein for immobilisation requires its exposure to both protecting and de-protecting agents, which may negatively impact its native structure and/or function.

Light-induced immobilization techniques have also been explored, leading to the use of quinone compounds for photochemical linking to a carbon-containing support (EP0820483). Activation occurs following irradiation with non-ionising electromagnetic radiation in the range from UV to visible light. Masks can be used to activate certain areas of the support for subsequent attachment of biomolecules. Following illumination the photochemically active compound, anthraquinone, will react as a free radical and form a stable ether bond with a polymer surface. Since anthraquinone is not found in native biomolecules, appropriate ligands have to be introduced into the biomolecule. In the case of proteins, this additional sample preparation step may require thermochemical coupling to the quinone and may not be site specific.

A further development of light-induced immobilisation technology is disclosed in US 6406844, which describes a method for preparing a linker bound to a substrate. The terminal end of the linker molecule is provided with a reactive functional group protected with a photo-removable protective group, e.g. a nitro-aromatic compound. Following exposure to light, the protective group is lost and the linker can react with a monomer, such as an amino acid at its amino or carboxy-terminus. The monomer, furthermore, may itself carry a similar photo-removable protective group, which can also be displaced by light during a subsequent reaction cycle. The method has particular application to solid phase synthesis, and does not facilitate orientated binding of proteins to a support.

A method for orientated, light-dependent, covalent immobilization of proteins on a solid support, using the heterobifunctional reagent N-[m-[3-(trifluoromethyl)diazirin-3-yl]phenyl]-4-maleimidobutyramine, is described by Collioud A *et al.* (1993) in *Bioconjugate Chem.* 4: 528-536. The aryldiazirine function of this cross-linking reagent facilitates light-dependent, carbene-

mediated, covalent binding to either inert supports or to biomolecules, such as proteins, carbohydrates and nucleic acids. The maleimide function of the cross-linker allows binding to a thiolated surface by thermochemical modification of cysteine thiols. Orientated binding of this cross-linking reagent to a protein can be attained by a thermochemical interaction between the maleimide function and an exposed thiol group on the protein surface, however this treatment may modify the structure and activity of the target protein. Light-induced covalent coupling of the cross-linking reagent to a protein via the carbene function, however, has the disadvantage that it does not provide for controlled orientation of the target protein.

Common for most of the described immobilisation methods is their use of one or more thermochemical/chemical steps, sometimes with hazardous chemicals, which are likely to have a deleterious effect on the structure and/or function of the bound protein. The available methods are often invasive, whereby foreign groups are introduced into a protein to act as functional groups, which cause protein denaturation, as well as lower its biological activity and substrate specificity.

There is a need in the art of protein coupling and immobilisation to improve the method of coupling, where the structural and functional properties of the coupled or immobilised component are preserved and the orientation of coupling can be controlled.

Summary of invention

The present invention provides a method for coupling proteins on a carrier via stable and strong bonds (covalent bond or thiol-Au bond) while preserving the native structural and functional properties of the coupled protein, and avoiding the use of one or more chemical steps. This contrasts with traditional coupling methods for protein immobilisation, which typically involve several, chemical reactions, which can be costly, time-consuming as well as deleterious to the structure/function of the bound protein. Furthermore the orientation of the proteins, coupled according to the method of the present

invention, can be controlled, such that their functional properties e.g. enzymatic, may be preserved. In comparison, the majority of known protein coupling methods lead to a random orientation of the proteins immobilised on a carrier, with the significant risk of lower biological activity and raised detection limits. The invention exploits an inherent property of proteins, whereby disulfide bridges in a protein, located in close proximity to aromatic amino acid residues, are disrupted following prolonged irradiation with light absorbed by these aromatic amino acids. The thiol groups created by light-induced disulfide bridge disruption in a protein are then used to immobilise the protein to a carrier.

According to a first aspect of the invention, a method of coupling disulfide bridge containing proteins on a carrier is provided, comprising the following steps of irradiating a protein creating a thiol group, and incubating the irradiated protein with a carrier capable of binding a thiol group. Coupling according to the invention is furthermore possible by first incubating the protein together with a carrier capable of binding a thiol group and thereafter irradiating the protein in the presence of the carrier to create a thiol group in the protein, and thereby obtaining a coupling to the thiol ligand binding group on the carrier. According to this method of coupling or immobilization, the irradiation comprises light of a wavelength which excites one or more aromatic amino acids. The irradiated aromatic amino acids in the protein comprise tryptophan, tyrosine and phenylalanine, or alternatively the aromatic amino acid is tryptophan. According to a further aspect of the invention, the irradiation comprises wavelengths in the vicinity of 295nm, 254nm or 275nm. According to a further aspect of the invention, the irradiated protein is coupled to a carrier that comprises a peptide or protein. Alternatively the irradiated protein is coupled to a support which comprises gold, or is derivatised with a thiol binding group, or comprises a thiol group or a disulfide bridge, where coupling to the support may be an immobilisation. Optionally the carrier or support comprises a spacer. According to a further aspect of the invention is provided a carrier or support comprising the coupled protein, produced according to the disclosed method of coupling

proteins on a carrier. According to a further aspect of the invention, the protein, coupled according to the disclosed method of coupling a protein on a carrier includes enzymes, transcription factors, protein domains, antigens, antibodies or binding proteins as well as other proteins. According to a further aspect of the invention the carrier, coupled according to the disclosed method of coupling a protein on a carrier, includes a pharmaceutical drug. According to a further aspect of the invention is the use of the carrier or support comprising the coupled protein, produced according to the disclosed method of coupling proteins on a carrier, for bio-functional assays. This use for bio-functional assays comprises: bio-sensors, chromatography, immunodetection, enzyme assays, nucleotide binding detection, protein-protein interaction, protein modifications, carrier targeting or protein targeting.

Brief description of drawings

Figure 1 shows the fluorescence intensity of cutinase at 350 nm as a function of time of illumination at 295 nm.

Figure 2 shows the Trp fluorescence (F) emission intensity increase of cutinase at 350 nm [F/Fo] as a function of time of illumination at 295 nm (solid line) and the concentration of newly formed free thiol groups in cutinase samples with specific ratios of fluorescence emission increase [F/Fo] (open circles).

Figure 3 shows the fluorescence emission spectrum of a 1μM cutinase solution upon excitation with 295 nm light during incubation (A) at 25°C without DTT [solid circles]; (B) at 70°C without DTT [solid black line]; (C) at 25°C without DTT, following heating to 70°C and cooling to 25°C [hollow circles]; (D) at 25°C with DTT, following heating to 70°C, addition of DTT and cooling to 25°C [solid grey line].

Figure 4 shows spectral characteristics of aromatic residues in aqueous solution.

Figure 5 shows the coupling reaction between an SH group of a protein and an SS group of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the stoichiometric release of nitrothiobenzoate (NTB).

Definitions

As used herein, the terms "UV light" or "irradiation" or UV illumination" or "UV irradiation" is a range of wavelengths or single wavelength of UV light, or IR/visible light for multiphoton excitation, that specifically excites aromatic amino acids, preferably a wavelength of approximately 295 nm, 275 nm or 254 nm, more preferably the wavelengths that excite tryptophan, tyrosine or phenylalanine, most preferably the wavelength, 295 nm, that excites tryptophan.

As used herein the term "protein" comprises polypeptides, fragments of proteins and antibodies or any other amino acid based material. Furthermore the term "protein" includes enzymes, antibodies, antigens, transcription factors, binding proteins e.g. DNA binding proteins, or protein domains.

As used herein, the term "spatial neighbour" relates to the physical distance between two chemical groups within a composition, such that groups lying in three-dimensional close proximity are considered to be spatial neighbours. A disulfide bridge in a protein that is a spatial neighbour to an aromatic residue may function as a quencher if the aromatic amino acid absorbs excitation energy following irradiation. The physical distance between half cystines of a disulfide bridge, which are spatial neighbours to tryptophan residues and may act as quenchers, can be, but is not limited, a range of 1 to 10 Å.

As used herein, the term "carrier" can be a soluble compound or polymer, or an insoluble compound or polymer, where the compound or polymer comprises a thiol-binding ligand, such as a reactive SH or an SS bond capable of coupling to an irradiation-induced thiol group. Furthermore the term "carrier" should be understood to include supports comprising material

capable of coupling to an irradiation-induced thiol group, such as a gold support or a derivatised support carrying a thiol-binding ligand.

As used herein, the term "support" can be any support material such as electronic chips, slides, wafers, particles, resins, wells, tubes or membranes which include but are not limited to any material comprising polymers such as Topaz, polystyrene, polyethylene, polyester, polyethermides, polypropylene, polycarbonate, polysulfone, polymethylmethacrylate [PMMA], Poly(vinylidene flouride) [PVDF], silicone; diamond; glass e.g. quartz and silica; silicium e.g. silicium wafers; metals; membranes e.g. nylon membranes, nitrocellulose filters; porous materials such as gels, agarose or cellulose; ceramics etc, which furthermore include all forms of derivatisation of the support which facilitate binding of thiol groups with or without intervening spacers.

As used herein, the term "spacer" comprises a chain of compounds with the purpose of providing a link between a protein and a carrier or raising an immobilised protein above the surface of a support.

As used herein, the term 'pharmaceutical drug' comprises articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and articles (other than food) intended to affect the structure or any function of the body of man or other animals and articles intended for use as a component of any article specified above.

As used herein the term "bio-functional assay" comprises a biosensor, immunodetection, an enzyme assay, nucleotide-binding detection, chromatography, protein-protein interaction, protein modification, carrier targeting or protein targeting.

As used herein, the term "biosensor" comprises an analytical devise incorporating biological or biologically-derived sensing elements, such as an amino acid (e.g., cysteine), protein, antibody, nucleic acid, microorganism, or

cell. The sensing element is either integrated within or intimately associated with a physicochemical transducer. The general aim of a biosensor is to produce either discrete or continuous digital electronic signals which are proportional to a single analyte or a related group of analytes.

Detailed description of the invention

The present invention exploits an inherent property of proteins, concerning irradiation-induced structural changes in proteins, thought to retard their photo-degradation. When proteins are exposed to UV irradiation some disulfide bridges are disrupted to form activated thiols. Although disulfide bridges are commonly found in the structural core and near/on the surface of folded proteins, those located in close proximity to aromatic amino acids are the most susceptible to UV-induced disruption. During UV exposure of proteins, energy absorbed by side chains of aromatic amino acid residues are transferred to neighbouring disulfide bridges, which function as quenchers (Neves-Petersen MT., *et al.*, 2002, *Protein Science* 11: 588-600). However, the flow of energy transferred to disulfide bridges ultimately serves to trigger their disruption. The presence of a disulfide bridge with tryptophan as a close spatial neighbour in a protein occurs frequently in nature, indicating that photo-induced disulfide bridge disruption is a widespread phenomenon (Petersen MTN., *et al.*, 1999, *Protein Engineering* 12: 535-548; Neves-Petersen MT *et al.*, 2002, *Protein Science* 11: 588-600; Vanhooren A *et al.* 2002, *Biochemistry* 10; 41(36):11035-43)."

The exploitation of this irradiation-induced phenomenon for the orientated immobilisation of proteins on a carrier is the basis for this invention. Cutinase, from the fungus *Fusarium solani pisi*, is chosen as a model protein to illustrate the process of light-induced disulfide bridge reduction and its immobilisation on a carrier, however the application of the invention is by no means limited to the model protein. Cutinase is a lipolytic enzyme capable of

degrading cutin, an insoluble lipid-polyester matrix found on the surface of plant leaves. Cutinase is an industrially important enzyme, and is incorporated in detergents for the removal of fats. It has two disulphide bridges; one near the active site, and one at the opposite pole of the protein, in close proximity to the single tryptophan residue of protein. Chemical reduction of the disulphide bridge, located near the active site, renders the enzyme inactive (Soliday CL, *et. al.*, 1983, *Biochem Biophys Res Commun* 114:1017-22).

In its native conformation the single tryptophan residue of *F. solani pisi* cutinase is highly quenched due to the presence of the adjacent disulfide bridge. Following prolonged selective irradiation of cutinase at 295nm, the fluorescence quantum yield of the single Trp residue increases simultaneously with the disruption of the neighbouring disulfide bridge. The increased quantum yield of the Trp residue in cutinase reflects the loss of the disulfide bridge and its ability to quench the Trp residue in an excited-state.

Disulphide bridges are known to be excellent quenchers of excited-state aromatic residues. Any aromatic residue, which is in close spacial proximity, can cause photo-induced disruption of a neighbouring disulfide bridge. Hence the three aromatic amino acids, tryptophan, tyrosine and phenylalanine found in proteins, are all potential mediators of light-induced disulfide bridge disruption. While irradiation with light of a range of wavelengths extending from 240nm to 300nm will excite all aromatic residues, the individual aromatic residues have differing absorption maxima (Table 1).

Table 1

In water	Absorption Max.	Emission Max.
Phe	254 nm	282 nm
Tyr	275 nm	303 nm
Trp	280 nm	250 nm

Since the excitation spectrum of the aromatic amino acid residues is only partially overlapping, protein irradiation at a single or narrow wavelength range will excite the individual residues to different degrees. Irradiation at 295 can be used to selectively excite tryptophan residues in a protein. Irradiation at 280nm will excite both tyrosine and tryptophan residues, which can both then cause photo-induced disulfide bridge disruption. Where irradiation is performed by multiple-photon excitation, for example when two-photon excitation is carried out, the sample is irradiated with photons (light) with half the energy (twice the wavelength) of the photons used in a single-photon experiment. For example, electronic excitation of tryptophan can both be achieved with ultraviolet light at 295 nm, or with two-photon excitation at a wavelength of approximately 690 nm. Furthermore excited tyrosine residues can cause the excitation of neighbouring tryptophan residues by a mechanism called fluorescence resonance energy transfer, which in turn can cause disulfide bridge disruption.

It will be apparent to those skilled in the art that the disruption of disulfide bonds in a given protein at a selected wavelength can be predicted from the location and amino acid neighbours of each disulfide bridge in the 3D structure of the protein. Disulphide bridges placed in the spatial vicinity of aromatic amino acid residues are likely to be the most labile to UV light. The 3D structures of a subset of proteins containing the triad Trp Cys-Cys, in close spatial proximity, have been examined in order to identify which amino acids are located in immediate vicinity of the tryptophan residues of the triad. This analysis has identified those proteins having a similar amino acid neighbourhood composition around the triad to that of cutinase, that can be used to predict which proteins will have the disulphide bond of the triad broken upon UV illumination. One such protein is goat α-lactalbumin, where the amino acid residues located within an 8 Å sphere around the tryptophan residue of the triads of goat α-lactalbumin are very similar to that of cutinase. Furthermore, It has recently been shown that upon UV excitation of goat α-

lactalbumin, those disulphide bridges lying adjacent to tryptophan residues are disrupted and free thiol groups are formed (Vanhooren A *et al.* 2002, *supra*). It is also possible to predict the UV stability of putative disulphide bridges in a given protein, solely on the basis of its primary structure, i.e., its amino acid sequence, provided that the 3D structure of homologous proteins is known and can be used for homology modelling of the 3D structure of the given protein.

It will also be apparent that known methods of recombinant DNA technology can be used to introduce amino acid substitutions into a protein sequence to create additional photo-disruptable disulfide bridges. Such substitutions may introduce a tryptophan residue in a protein as close spacial neighbour of endogenous disulfide bridge, or alternatively two cysteine residues may be introduced in close spacial proximity to an endogenous tryptophan residue. Alternatively both tryptophan and two cysteine residues may be introduced in close spacial proximity to each other. Irradiation with 295 nm light is preferable since it permits the selective excitation of tryptophan residues in a protein, which in turn may lead to the disruption of a single or a limited number of disulfide bonds. A variety of light sources suitable for the irradiation of proteins at a range of wavelengths, for the photo-induction of disulfide bond disruption include, but are not limited to, a 75-W Xenon arc lamp from a research grade spectrometer such as a RTC PTI spectrometer, a deuterium lamp, a high pressure mercury lamp. Irradiation at a single wavelength can be obtained by coupling the light source to a monochromator. A source of multiple photon excitation includes a high peak-power pulsed laser.

New thiol groups are formed in proteins following light-induced disulfide bond disruption. Their appearance *de novo* can be measured by a 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB] based assay. In the case of cutinase, irradiated at 295nm, the formation of one thiol group per illuminated protein was detected

on average. There are no free thiols in native cutinase, and disruption of the disulfide adjacent to the single tryptophan residue only yields one solvent accessible thiol that can be detected by this method. Light-induced thiol groups formed on proteins, which are accessible, will bind to any thiol binding ligand or free thiol group on a carrier.

This method of coupling to a carrier can be used to construct various types of disulfide-linked oligomers or polymers. Light-induced thiol groups in a given protein or peptide can be coupled to a carrier comprising a peptide or protein. Provided that the concentration of protein and carrier molecules in the coupling reaction is sufficiently high, SS based cross-linking between neighbouring molecules will take place. While the light induced protein should contain an SS bridge, the possession of an aromatic residue as close spacial neighbour is not essential, since the aromatic contribution to the reaction may be supplied by aromatics residues added to the coupling reaction.

This method of light-induced thiol coupling to a carrier can be usefully applied to other types of carrier molecule, such as pharmaceutical drugs, in order to facilitate their effective delivery. For example, light-induced thiol coupling of a water-soluble molecule containing a disulfide bridge (including but not limited to a peptide or protein) to a drug can help the solubilisation and delivery of water-insoluble, poorly soluble or hydrophobic drugs. Furthermore the molecule coupled to the drug may serve to protect the drug from its physiological environment, and hence improve its stability *in vivo*. This particular feature makes this technology attractive for the delivery of labile drugs such as proteins. Localized delivery of the molecule coupled drug, by implantation at the site of treatment, would reduce systemic exposure of the patient to the drug. Carrier-linked prodrugs are generally defined as prodrugs that contain a temporary linkage of a given active substance to a transient carrier group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic

cleavage. In the present invention, light-mediated disruption of the disulfide bond linking a drug to a molecule can be used to achieve a controlled release of the active drug from the molecule-coupled form, implanted in the patient. This would minimise the frequency of drug delivery to the patient, and provide for light-controlled dosing. This feature would improve patient compliance, especially for drugs used for chronic indications, requiring frequent injections (such as for deficiency of certain proteins or metabolites). Controlled drug release could be induced by infra-red light (via two-photon excitation) in the case of transdermal drug delivery, within the penetration range of infra-red light, while the greater penetration of UV light (or infra-red light via three-photon excitation) would facilitate drug release deeper within the patient. Since a solvent exposed disulfide bridge will be broken in a reducing environment the drug could also be released when the carrier coupled with the drug has entered a reducing environment such as the cytoplasmic space of a cell.

This method of light induced thiol coupling can also be used to immobilise a protein on a support. The most common types of bonds that are formed during coupling to a support are disulfide bonds and sulfur-metal bonds (primarily sulfur-gold) where a self-assembled layer is formed. As both types of bonds are strong and stable, extensive washing after immobilization will not displace the protein. The density of proteins on a support can be controlled by varying the protein concentration, and subsequently blocking the remaining activated thiol groups on the surface with reagents such as L-cysteine, (2-(2-pyridinyldithio)ethaneamine hydrochloride (PDEA) or with a thiol-lipid bilayer (Hong Q., et. al., 2001, *Biochemical Society Transactions* 29(4):587-582). The support, with evenly distributed immobilized proteins, is therefore blocked to prevent non-specific binding. According to the present invention the method of immobilization does not involve any chemical steps, since the thiol-activated proteins formed by UV radiation, spontaneously self-

assemble on the support. The described thiol and disulfide exchange reactions are an effective and rapid way to bind molecules to supports.

Immobilisation of a protein on a support can also be spacially controlled. Present day laser technology allow for focal spots with dimensions of 1 micrometer or less. If a specific protein or target molecule, containing SS bridge(s), is incubated with a thiol-binding support, light-induced thiol group formation and coupling could be limited to the focal points of illumination. The viscosity of the solution should be controlled to minimise diffusive processes that might disperse the illuminated molecules beyond the spot size. This approach would allow for an extremely dense packing of identifiable and different molecules on a support surface. Thus the method of the present invention could be used for charging microarrays with molecules.

In a further aspect of the present invention, the orientation of the immobilized protein can be controlled in a uniform and reproducible manner. Prolonged selective excitation of tryptophan residues in a protein will only lead to disruption of those disulfide bridges to which excitation energy is transferred. The location of these photo-disruptable disulfide bridges, forming free thiol groups, can be predicted from the protein's structure, where it is known from three-dimensional models, NMR or crystallography. In those cases where only a single thiol group is induced by irradiation of tryptophan residues in a protein, as is the case for cutinase, then immobilisation of the protein on a support will occur exclusively via this thiol group. In contrast to alternative methods of disulfide bridge disruption and thiol-group formation, such as the use of reducing agents, the light-induced method of the present invention leads to targeted disruption of disulfide bridges forming one or only a few accessible thiol groups, whose position can be precisely predicted. The subsequently immobilised proteins will thus have a single or very limited number of orientations. Since cutinase has only a single thiol group for immobilisation, which is distant from the active site, the accessibility of

substrates will not be limited by immobilisation. Immobilisation via a surface accessible thiol group, remote from the protein's active site, as is the case of for cutinase, is also less likely to alter the conformation or structural properties of the protein. In other words, the immobilisation method of the present invention serves to preserve the native state of the immobilised protein. All functional/structural assays performed on proteins, which are immobilised in a uniform orientation according to the methods of the present invention, will generate data derived from a uniform population of proteins. The structural and functional uniformity of the immobilised proteins, and retention of their native state, is of primary importance for screening or assaying proteins for catalytic, binding, or any other biological properties and provides one of the many valuable advantages of the present invention.

In a further aspect of the present invention the bond immobilising the protein to the support can be disrupted, releasing the protein into solution. This is possible for both disulfide bridges, as well as thiol-metal bonds. Disulfide bridges between a protein and a support can be disrupted e.g. with UV irradiation, in the same way as disrupting a disulfide bond on a protein, where an aromatic amino acid is a spatial neighbour. The aromatic amino acid can either be located on the immobilised protein itself, or be supplied in the form of a solution of an aromatic amino acid, such as tryptophan, applied to the support surface. Alternatively, disulfide bridges between a protein and a support can be disrupted with (dithiothreitol) DTT, or other reducing agents known to persons skilled in the art. Following disruption of the immobilisation bond, the released protein can be purified, if necessary, and used in further experiments.

A further aspect of the present invention is regenerating a gold surface by removing proteins that are immobilized through a thiol-Au bond with O_2-plasma treatment or Piranha, thereby removing the top layer of the gold surface including the proteins.

Examples

Example 1: Formation of free thiol groups in cutinase upon UV illumination

The disruption of disulfide bridges in a protein following UV illumination was examined using a cutinase, with lipase/esterase properties isolated from *Fusarium solani pisi.*

Steady-sate fluorescence emission intensity of cutinase

Cutinase preparations were subjected to UV irradiation at 295 nm under the following conditions in order to follow its steady-state fluorescence emission intensity with time: Three ml of 2μM stock solution of cutinase was continuously illuminated at 295 nm for increasing periods of time (0h, 1h, 2h, 3h, 4h, and 5h) in a quartz macro-cuvette (1 cm path length). Light excitation at 295 nm was supplied by a Xenon Arc Lamp coupled to a monochromator provided by a RTC 2000 PTI spectrometer. The cuvette was mounted in a thermostated cuvette, kept at a constant temperature of 25°C. The cutinase sample was maintained as a homogeneous solution by continuous stirring at 700 rpm with a magnet. Excitation and emission slits were set at 6 nm. The fluorescence intensity of cutinase at 350 nm was monitored throughout 5h of illumination.

The time dependent fluorescence intensity of a 2μM cutinase solution at 350 nm upon illumination with 295nm light is shown in Figure 1. Fluorescence intensity increased very rapidly over the first 7200s, followed by a plateau where the fluorescence yield appeared to stabilise.

Detection of free thiols in cutinase following UV illumination

The concentration of free thiol groups in cutinase formed following UV illumination was determined as follows: Thiol groups on the cutinase were detected and quantified by a spectrophotometric assay based on the reaction

of thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) [DTNB] or Ellman's reagent (Ellman GG,. 1959 *Arch. Biochem. Biophys.* 82: 70-77; Hu ML., 1994, *Meth. Enzymology* 233: 380-385). Three ml of a 17.3µM solution of cutinase in 20mM TrisHCl pH 8.5 was illuminated with 295nm light in a quartz macro-cuvette (1 cm path length) for different periods of time using a RTC 2000 PTI spectrometer, as described above. The time dependent fluorescence emission intensity of the sample at 350nm was measured. All slits were set to 2 nm band-width. An excess of DTNB (100µl of an 8.5mM DTNB stock solution in absolute methanol) was added to 900µl of cutinase solution, prior to or after its illumination at 295 nm. The stock solution of DTNB in methanol is stable for up to 2 weeks at 4°C (Hu ML., 1994 *supra*). Immediately after mixing the two components, absorbance of the released NTB ion (nitrothiobenzoate ion, ε_{412nm}=13600$M^{-1}cm^{-1}$) was measured at 412 nm with a UV/Visible Pharmacia spectrophotometer, and again after 20 and 24 min reaction time at 25°C. The free thiol concentration is proportional to the absorbance value at 412 nm. The readings were stable between 20 and 24 min. Each data point was an average of three measurements after 24 min. The control sample comprised 100µl of a 8.5mM DTNB stock solution in absolute methanol mixed with 900µl of non-irradiated cutinase (17.3µM cutinase solution in 20mM TrisHCl pH 8.5). The concentration of illuminated cutinase sample was raised to ensure that the amount of free thiol groups formed upon illumination were above the detection limit of the DTNB method.

Free thiol groups were not detected in non-illuminated control samples of cutinase using the DTNB assay. This is consistent with the absence of free thiol groups in native cutinase, since all four cysteine residues in the enzyme are involved in disulphide bridges. The amount of thiol groups formed in the cutinase sample during illumination was measured as a function of the fluorescence intensity increase (F/Fo). Figure 2 demonstrates a positive correlation between the time-dependent increase in Trp fluorescence emission intensity at 295 nm of cutinase upon UV excitation, and the

increased concentration of free thiol groups detected at different ratios of fluorescence emission increase (F/Fo).

These results support the working model that the initial fluorescence intensity of tryptophan (Trp) is highly quenched due to the presence of a nearby intact disulfide bridge, and that the fluorescence intensity increase of the single endogenous Trp in cutinase is due to the cleavage of the nearby disulfide bridge upon illumination of cutinase at 295 nm.

Example 2: Irradiation-induced disruption of a disulfide bridge in native cutinase is dependent on the presence of a Trp residue as spatial neighbour

In order to demonstrate the role of a tryptophan residue in cutinase in the irradiation induced disruption of a disulfide bridge, the Trp fluorescence emission intensity of the native protein was compared to that of reduced cutinase in which all disulfide bridges were chemically disrupted. The cutinase protein was partially denatured by heat in order to facilitate reduction of all its disulfide bridges. The Trp fluorescence emission intensity of the native and denatured cutinase, following irradiation, was used to demonstrate the importance of a Trp residue being a special neighbour of a disulfide bridge, for the transfer of excitation energy from Trp to the disulfide bridge.

Reduction of native cutinase with DTT

In native cutinase, the disulfide bridges within the folded protein are inaccessible to solvent, and they cannot be directly reduced by DTT. However, if the cutinase is heated in a pH 8.5 buffer to a temperature exceeding its unfolding temperature, the subsequent unfolding process will facilitate the access of DTT to the protein's disulphide bridges. The heat denaturation step was performed on a dilute solution of cutinase, in order to avoid precipitation of the protein. The buffer (20mM Tris-HCl 8.5),

selected for the heat denaturation of cutinase, displays a minimal pH drift in relation to temperature and volume changes, and furthermore Tris-HCl has minimal enthalpy of ionisation. The concentration of cutinase solutions was estimated from the OD 280nm of the solution, using the extinction coefficient of cutinase at 280nm (13500 $M^{-1}cm^{-1}$).

Reduction of cutinase disulfide bonds with DTT was performed as follows: A 650µl sample of 1µM cutinase suspended in 20mM TrisHCl pH 8.5 was heated from 25°C to 70°C, to which excess DTT was added (8.5 µl of 3.7M DTT in 20mM TrisHCl pH 8.5), to give a final concentration of 50mM DTT.

Trp fluorescence emission intensity measurements of native or reduced cutinase

Emission spectra of 1µM cutinase samples, upon excitation at 295 nm, were measured under the following conditions with a RTC 2000 PTI spectrometer, with slits set at 2 nm band width: (A) cutinase incubated at 25°C without DTT; (B) cutinase incubated at 70°C without DTT; (C) cutinase incubated at 25°C without DTT, following heating to 70°C and cooling to 25°C; (D) cutinase incubated at 25°C with DTT, following heating to 70°C, addition of DTT and cooling to 25°C. The spectra were corrected for Raman contribution.

The four emission spectra are shown in Figure 3. The emission spectrum of cutinase incubated at 25°C (A) is the same as the emission spectrum of cutinase after cooling from 70°C to 25°C (C), demonstrating that thermal unfolding of cutinase at pH 8.5 is a reversible process. This can be compared with the emission spectrum of cutinase in an unfolded state (B). The Trp emission fluorescence intensity of cutinase at 25°C, after heating to 70°C in the presence of DTT (D) was greater than for native cutinase without DTT, as in (A). These spectra serve to confirm the correlation between an increase in Trp fluorescence emission intensity of cutinase

(upon excitation at 295nm) and reduction of its disulfide bridges, shown in Example 1.

In the course of obtaining spectra for samples (A) as well as (C), with excitation of the Trp residue of cutinase at 295nm, the fluorescence emission was observed to increase, as shown in Example 1. The similar fluorescence increase in (A) and (C) is consistent with the close proximity between the Trp and the disulphide bridge observed in the 3D structure of native cutinase upon refolding. This increase in fluorescence emission, upon excitation of the Trp residue of cutinase at 295 nm, was not observed for samples (B) or (D). These data support the conclusion that a close contact between the Trp residue and the disulphide bridge in cutinase is necessary for the photo-induced mechanism, and that this proximity is lost when the protein is unfolded at 70°C, pH 8.5. This is the most likely reason why the Trp fluorescence quantum yield of cutinase at 70°C was higher than at 25°C, despite the fact that one would expect a lower quantum yield due to the higher temperature causing collisional quenching between the Trp and solvent molecules.

Example 3. Specificity of light induced disulphide bridge disruption

The cutinase gene of *Fusarium solani pisi* has been mutated to encode a cutinase polypeptide where the single tryptophan residue is replaced by alanine, a non-fluorescent amino acid. The mutant cutinase is expressed and a solution of the protein is illuminated with light at 296 nm. Tryptophan is the only aromatic amino acid residue to be excited by light at a wavelength of 296 nm. The absorption (A) and emission spectra (F) of the aromatic amino acids in aqueous solution at pH7 is shown in Figure 4. In contrast to the native wild-type cutinase, the mutant will not absorb 296nm light, since the mutant lacks tryptophan, and the tyrosine (if protonated, favoured approximately at pH 4) and phenylalanine residues do not absorb light of this wavelength. In the absence of Trp excitation in the mutant cutinase, the disulfide bridge next to the inserted alanine remains intact, and no free SH

group is detected upon 296 nm illumination. These data demonstrate that the disulfide bridge in close proximity to the tryptophan amino acid of cutinase is disrupted following illumination.

The identity of the disrupted disulfide bridge in cutinase following UV illumination is confirmed by further studies. The native cutinase is known to have two disufide bridges, one of which is located close to the active site and is essential for catalytic activity. Since the native cutinase was found to retain its enzymatic activity following UV illuminated, it is evident that only the surface localised disufide bridge is disrupted. The formation of DTNB detectable thiol groups in native cutinase following UV irradation provides an additional tool for quantitating the disruption of disufide bridges. Following UV illumination only one thiol group per cutinase molecule was detected. This is consistent with the disruption of the surface localised disulfide bond, where the second thiol group is not solvent accessible. At least one of the cystine residues of the second disulfide bridge is solvent accessible, but disruption was not detected.

Example 4. Light induced cutinase coupling.

The formation of free SH groups in a model protein, namely cutinase from *Fusarium solani pisi*, in response to illumination has been shown in Examples 1 and 2. The reactive thiol group of the cutinase can then be coupled to carrier molecules in solution which have either a free and solvent-accessible thiol group or a solvent accessible disulfide bridge. The method of light induced cutinase coupling to carrier molecules in solution is illustrated by the coupling of a free thiol goup in cutinase, induced by irradiation with light of 295nm, to a disulfide group of DTNB, as shown in figure 5.

The coupling reaction was performed as follows. Three ml of a 17.3µM solution of cutinase in 20mM TrisHCl pH 8.5 was illuminated with 295nm light in a quartz macro-cuvette (1 cm path length) for different periods of time using a RTC 2000 PTI spectrometer, as described in example 1 and 2. An excess of DTNB (100µl of an 8.5mM DTNB stock solution in absolute methanol) was added to 900µl of cutinase solution, prior to or after its

illumination at 295 nm. Immediately after mixing the two components, absorbance of the released NTB ion (nitrothiobenzoate ion, ε_{412nm}=13600$M^{-1}cm^{-1}$) was measured at 412 nm with a UV/Visible Pharmacia spectrophotometer, and again after 20 and 24 min reaction time at 25°C. The coupling reaction with DTNB results in the formation of a stoichiometric amount the NTB ion. Thus, the concentration of coupled cutinase is proportional to the absorbance value at 412 nm. The control sample comprised 100µl of an 8.5mM DTNB stock solution in absolute methanol mixed with 900µl of non-irradiated cutinase (17.3µM cutinase solution in 20mM TrisHCl pH 8.5).

Example 5. Light induced cutinase immobilisation.

The free reactive thiol groups formed in cutinase from *Fusarium solani pisi*, in response to illumination, as shown in Examples 1 and 2, can then be used to link the cutinase to a thiol reactive support, whether it be gold, or gold derivatised with thiol groups, or quartz surfaces derivatised with SH groups or a polymer support derivatised with SH groups.

A polymer support is derivatized with SH groups by first activating with NHS/EDC which modifies carboxymethyl groups to N-Hydroxysuccinimide esters. A thiol group is then introduced on the support by incubation with cysteamine in 0.1 M borate buffer pH 8.0 and subsequently with DTT or DTE (Dithioerythritol) in 0.1M borate buffer pH 8.0. The support is flushed with 0.1M borate buffer pH 8.0 prior to immobilization.

A quartz support is derivatized with SH groups by first cleaning the quartz support to remove all contaminants. Thereafter hydroxyl groups are generated on the support for subsequent formation of a silanized layer containing reactive groups. Finally the support is cured, where the curing cross-links the free silanol groups.

In this example the cutinase is immobilized on thiol-activated quartz by illuminating the protein with UV light and allowing the newly formed SH groups to react with free SH groups available on the surface of a quartz slide surface, according to the following steps:

1. Derivatising a quartz slide surface with SH groups
    a) incubating the slide with chromic acid (80 g/L $K_2Cr_2O_7$ conc. H_2SO_4) for 30 minutes at 70-75°C, and
    b) treating the surface of the slide with 5% potassium persulfate ($K_2S_2O_8$) for 1 hour at 100°C, and
    c) dispensing approximately 10 mL/cm^2 of 0.03% (3-mercaptopropyl)trimethoxysilane (MPTS) in xylene on the surface, and then evaporating the solvent to yield a uniform, optically-perfect silane coating, and
    d) curing for 1 hour at 100°C.
2. Applying a 2 µM UV-irradiated cutinase solution to the derivatised quartz slide.
3. Incubating the slide overnight at 4°C.
4. Purging the slide with buffer (20 mMTris-HCl, pH 8.5) to remove unbound protein.
5. Inserting the quartz slide in a Total Internal Reflection Fluorescence (TIRF) apparatus to detect the number of bound protein molecules and thus the efficiency of immobilization.

During the incubation step, the solvent accessible, free SH groups of the cutinase react, upon contact, with the free SH groups of the derivatised surface of the quartz slide, and establish a disulfide bridge between the protein and the slide surface. Since a disulfide bridge is a covalent bond, the purging step with buffer solution does not displace the covalently bound molecules from the slide. The only covalent bonds linking the cutinase to the surface are via SH groups on the protein. Cutinase that has never been illuminated by UV light, and thus is devoid of free SH groups, provides a control for the SH group mediated immobilisation process. Although cutinase

adsorption to the slide surface occurs, it is washed off the slide surface during purging. Finally the efficiency and stability of light-induced, SH group mediated immobilisation is monitored by TIRF.

Example 5. Enzymatic activity of immobilized cutinase

The cutinase enzyme, disulfide bonded to a thiol reactive surface by light induced immobilisation, is shown to retain its full activity. The active site of cutinase is very remote from the tryptophan residue and the immobilising disulfide bridge, and hence it is likely to be solvent accessible, allowing access to substrate.

The activity of the immobilised cutinase is followed by fluorescence spectroscopy, whereby substrate solution is deposited on the immobilised cutinase slide and the increase in fluorescence due to product formation is measured according to the following steps:

1. Depositing 500 µl of 100 µM 4-methylumbelliferyl butyrate substrate solution on the surface of a slide with immobilized cutinase.
2. Incubating the slide at room temperature for 30 minutes.
3. Transferring the reaction mixture to a quartz cuvette.
4. Transferring the cuvette to a UV/VIS spectrophotometer.
5. Selecting an excitation wavelength of 365 nm and measuring the fluorescence emission at 445 nm and determining the amount of 4-methylumbelliferone released.

The substrate, 4-methylumbelliferyl butyrate, is dissolved in DMSO, yielding a 25.2 mM solution. 250 µl of the DMSO solution is added to circa 9 ml of 50 mM, Tris-HCl pH 7.5, which contains 0.6% Triton X-100. The volume is thereafter diluted to 10.5 ml. Cleavage of the ester bond in the non-fluorescent 4-methylumbelliferyl butyrate, by a lipase/esterase releases 4-methylumbelliferone, which fluoresces in the blue region of the visible

spectrum. Quantification of the released fluorescence gives a measure of enzyme activity.

Example 6. Reversible immobilization with UV light

UV light is used to release the cutinase enzyme, disulfide bonded to a thiol reactive surface by light-induced immobilisation. When tryptophan residues and disulfide bridges are present in solution, the irradiation of the tryptophan disrupts the disulfide bridge by a photo-induced mechanism. The immobilised cutinase is released by UV light irradiation according to the following steps:

1. Placing the slide with immobilized cutinase in a TIRF apparatus and monitoring the adsorbed protein concentration on the slide by fluorescence emission at 350nm.
2. Purging the slide with a continuous flow of 20mM Tris-HCl pH8.5 buffer containing 20 μM tryptophan.
3. Irradiating the slide with 295nm light for 5 hours.
4. Purging the slide with 20mM Tris-HCl pH8.5 buffer.
5. Measuring fluorescence of the slide at 350nm continuously, to follow protein elution.

Example 7. Reversible immobilization with reducing agent

The cutinase enzyme, disulfide bonded to a thiol reactive surface by light-induced immobilisation, is released by chemical reduction of disulfide bonds. Thereafter, the protein is dialysed in a 20mM Tris-HCl pH8.5 buffer, free of reducing agents, to regain its native structure. The immobilised cutinase is released by a reducing agent according to the following steps:

1. Placing the slide with immobilized cutinase in a TIRF apparatus and monitoring the adsorbed protein concentration on the slide by fluorescence at 350nm.
2. Purging the slide with a continuous flow of buffer (20mM Tris-HCl pH8.5 containing 50mM DTT) for 5 hours.

3. Purging the slide with 20mM Tris-HCl pH8.5 buffer.
4. Measuring fluorescence of the slide at 350nm continuously, to follow protein elution.

Claims

1. A method of coupling a disulfide bridge containing protein to a carrier comprising the following steps,
   a) irradiating said protein creating a thiol group,
   b) incubating the irradiated protein with a carrier capable of binding a thiol group and thereby obtaining a coupling,

   or

   1) incubating said protein with a carrier capable of binding a thiol group,
   2) irradiating the protein in the presence of said carrier to create a thiol group in the protein and thereby obtaining a coupling.

2. A method according to claim 1, wherein said irradiation step comprises light of a wavelength which excites one or more aromatic amino acids.

3. A method according to claim 2, wherein said aromatic amino acids comprise tryptophan, tyrosine and phenylalanine.

4. A method according to claims 2 or 3, wherein said irradiation comprises light with a wavelength of 295nm, 275nm or 254nm.

5. A method according to claim 3, wherein said aromatic amino acid is tryptophan.

6. A method according to any one of claims 2, 3 or 5, wherein the wavelength is 295nm.

7. A method according to any one of claims 1 to 6, wherein said carrier comprises a peptide or a protein.

8. A method according to any one of claims 1 to 6, wherein said carrier is a support.

9. A method according to claim 8, wherein said coupling is an immobilization on said support.

10. A method according to claim 9, wherein said support comprises gold.

11. A method according to claim 9, wherein said support is a derivatised support which binds a thiol group.

12. A method according to claim 9, wherein said support comprises a thiol group or a disulfide bridge.

13. A method according to claim 12, wherein the support comprises a spacer.

14. A carrier comprising one or more proteins coupled by the method of any one of claims 1 to 6.

15. A carrier comprising one or more proteins according to claim 14, wherein the carrier is a support.

16. A carrier according to claim 14 or 15, wherein the one or more proteins are selected from the group consisting of enzymes, transcription factors, protein domains, binding proteins, antigens and antibodies.

17. A carrier comprising one or more proteins according to claim 14, wherein the carrier comprises a pharmaceutical drug.

18. Use of a carrier according to any one of claims 14, 15 or 16 for a bio-functional reaction.

19. Use of a carrier according to claim 18, wherein said bio-functional reaction are selected from the group consisting of a bio-sensor, chromatography, immunodetection, enzyme assay, nucleotide binding detection, protein-protein interaction, protein modification, carrier targeting and protein targeting.

Abstract

The present invention involves a method of coupling disulfide bridge containing proteins to a carrier by inducing the formation of thiol groups on a protein with irradiation, and coupling the protein to the carrier. The formation of thiol group(s) in the protein is a consequence of the disruption of disulfide bridges following electronic excitation of aromatic amino acid residues located in close spacial proximity to the disulfide bridge. The light-induced coupling method facilitates the orientated immobilization of a protein on a carrier.

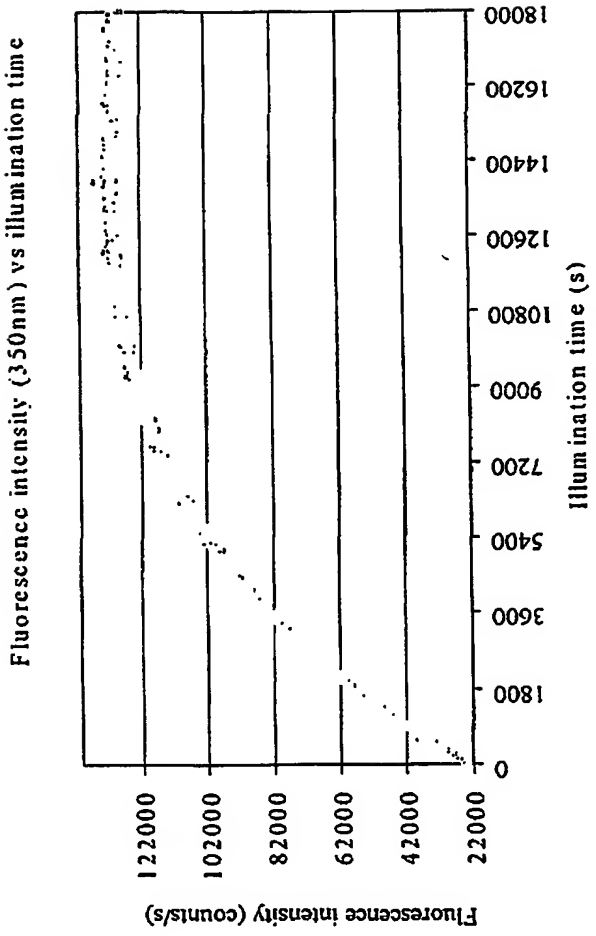

Figure 1

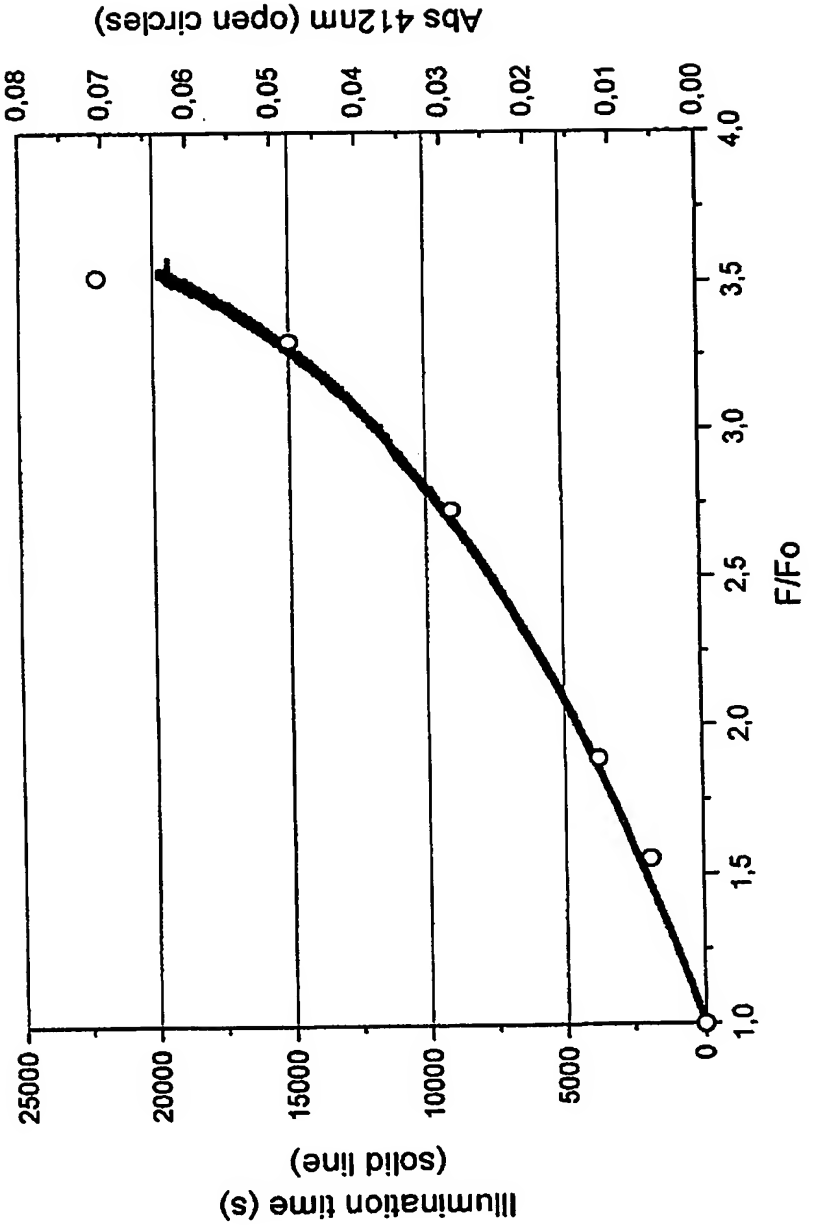

Figure 2

Figure 3

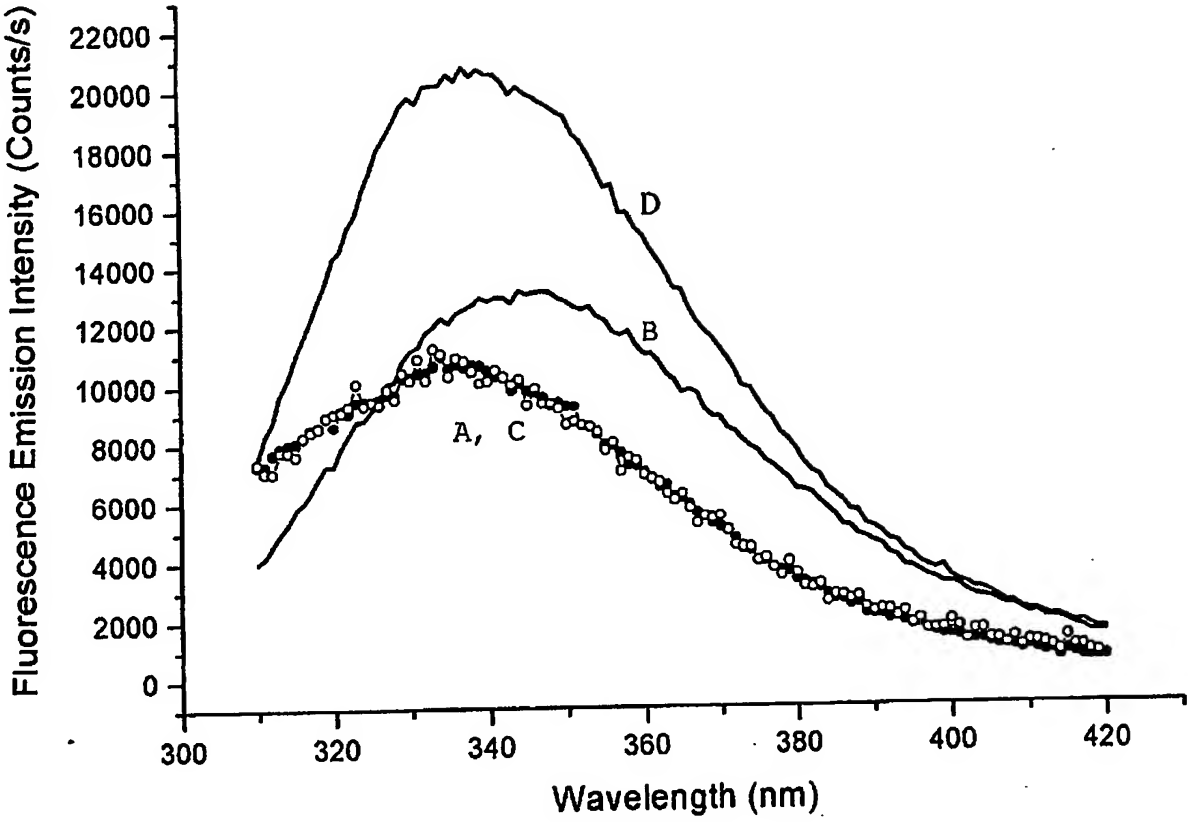

Figure 4

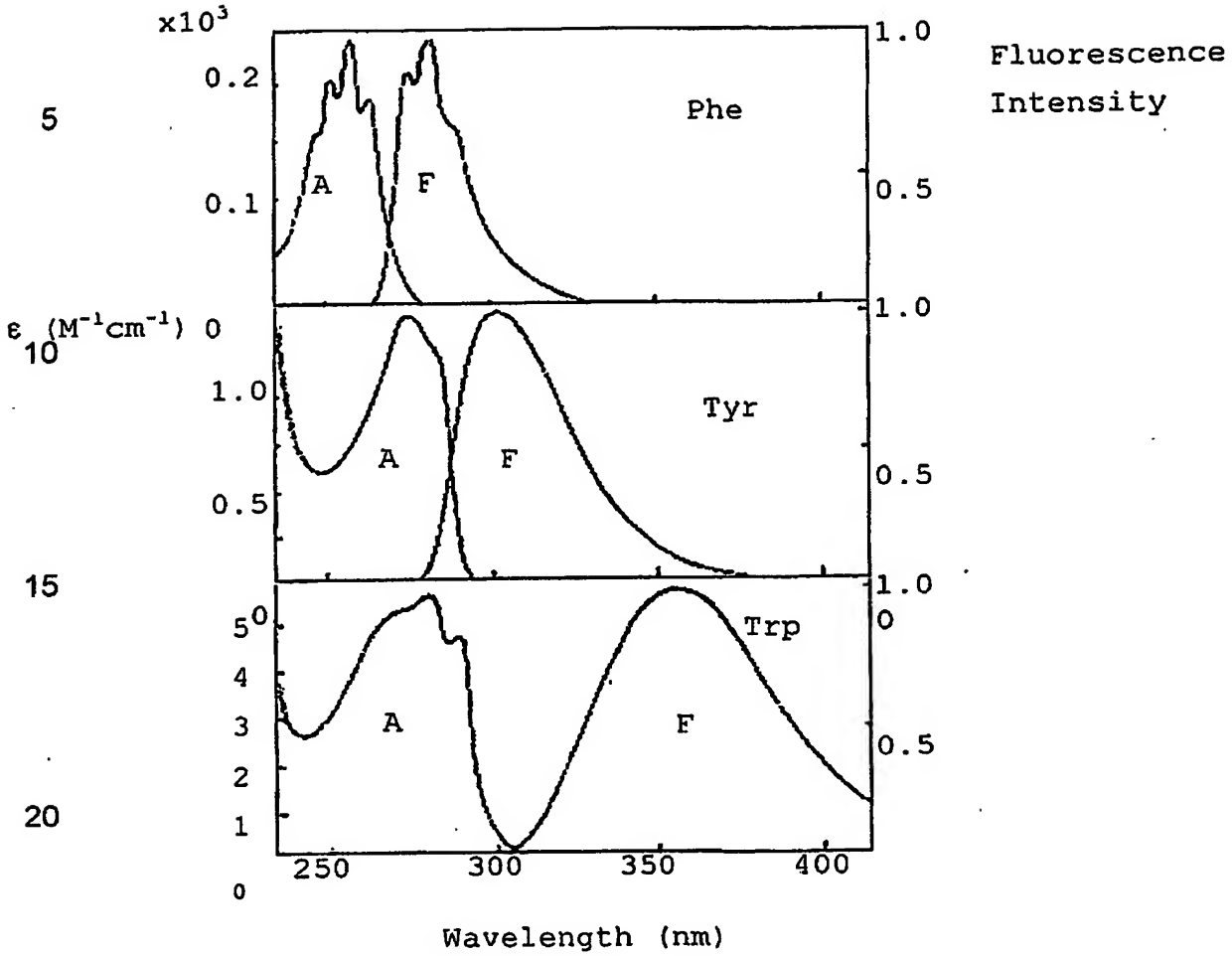

Figure 5